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Dietary administration of diquat for 13 weeks does not result in a loss of dopaminergic neurons in the *substantia nigra* of C57BL/6J mice

Daniel J. Minnema [a, *], Kim Z. Travis [b], Charles B. Breckenridge [a], Nicholas C. Sturgess [b], Mark Butt [c], Jeffrey C. Wolf [d], Dan Zadory [d], Mark T. Herberth [e], Scott L. Watson [f], Andrew R. Cook [b], Philip A. Botham [b]

[a] Syngenta Crop Protection, LLC, P.O. Box 18300, Greensboro, NC, 27419-8300, USA
[b] Syngenta Limited, Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, UK
[c] Tox Path Specialists, LLC, 8420 Gas House Pike, Frederick, MD, 21701-2607, USA
[d] Experimental Pathology Laboratories, Inc., 45600 Terminal Drive, Sterling, VA, 20166, USA
[e] WIL Research, Ashland, OH, 44805, USA
[f] RTI International, 3040 Cornwallis Road, Research Triangle Park, NC, 27709-2194, USA

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ABSTRACT

Male and female C57BL/6J mice were administered diquat dibromide ($DQ \cdot Br_2$) in their diets at concentrations of 0 (control), 12.5 and 62.5 ppm for 13 weeks to assess the potential effects of DQ on the nigrostriatal dopaminergic system. Achieved dose levels at 62.5 ppm were 6.4 and 7.6 mg DQ (ion)/kg bw/day for males and females, respectively. A separate group of mice was administered 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) ip as a positive control. The comparative effects of DQ and MPTP on the *substantia nigra* pars compacta (SNpc) and/or striatum were assessed using neurochemical, neuropathological and stereological endpoints. Morphological and stereological assessments were performed by investigators who were "blinded" to dose group. DQ had no effect on striatal dopamine concentration or dopamine turnover. There was no evidence of neuronal degeneration, astrocytic or microglial activation, or a reduction in the number of tyrosine hydroxylase positive (TH^+) neurons in the SNpc or neuronal processes in the striatum of DQ-treated mice. These results are consistent with the rapid clearance of DQ from the brain following a single dose of radiolabeled DQ. In contrast, MPTP-treated mice exhibited decreased striatal dopamine concentration, reduced numbers of TH^+ neurons in the SNpc, and neuropathological changes, including neuronal necrosis, as well as astrocytic and microglial activation in the striatum and SNpc.

1. Introduction

Diquat (6,7-dihydrodipyrido[1,2-a:2′,1′-c]pyrazinediium; DQ) and paraquat (1,1-dimethyl-4,4-bipyridinium; PQ) are bipyridyl herbicides. Based on structural similarities between PQ and the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), it has been proposed that exposure to PQ causes a loss of dopaminergic neurons in the *substantia nigra* pars compacta (SNpc) similar to the loss noted following MPTP exposure (McCormack et al., 2002). Although it is unlikely that PQ could produce neurotoxicity through the same mechanism as described for MPTP (Miller, 2007; Ramachandiran et al., 2007; Richardson et al., 2005), there are several published studies indicating that intraperitoneal (ip) doses of paraquat administered at weekly intervals result in the loss of dopaminergic neurons in the SNpc in young adult C57BL/6J male mice (Jiao et al., 2012; McCormack et al., 2002; Peng et al., 2004). However, the reliability of those results has recently been called into question by studies in which a detailed and blinded systematic analysis of neurochemical, histopathological and stereological endpoints failed to find any effect of PQ treatment at near-lethal acute ip doses

* Corresponding author. Syngenta Crop Protection, LLC, 410 Swing Road, Greensboro, NC, 27320, USA.
E-mail addresses: daniel.minnema@syngenta.com (D.J. Minnema), kim.travis@syngenta.com (K.Z. Travis), charles.breckenridge@syngenta.com (C.B. Breckenridge), nick.sturgess@syngenta.com (N.C. Sturgess), mbutt@toxpath.net (M. Butt), jwolf@epl-Inc.com (J.C. Wolf), dzadory@epl-Inc.com (D. Zadory), mark.herberth@wilresearch.com (M.T. Herberth), slwatson@rti.org (S.L. Watson), andy.cook@syngenta.com (A.R. Cook), phil.botham@syngenta.com (P.A. Botham).

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(Breckenridge et al., 2013; Smeyne, 2015) or following 13 weeks of continuous administration in the diet of C57BL/6J mice (Minnema et al., 2014).

DQ has also been reported to cause neurotoxicity in mice. Karuppagounder and colleagues reported that young adult, male C57BL/6 mice administered 10 mg/kg DQ ip, twice weekly for six weeks, had reduced numbers of tyrosine-hydroxylase-positive (TH^+) neurons in the SNpc (Karuppagounder et al., 2012). Unfortunately, that study did not provide any quantitative stereological data or histopathological confirmation of such effects. Furthermore, the actual amount of DQ that reaches the brain would be expected to be relatively low, based on the current findings as well as those of Lindquist (Lindquist et al., 1988). Lindquist and colleagues found that, following a single ip dose of radioactive DQ, the concentration of [^{14}C]DQ in mouse brains was 0.51% of the administered dose/g tissue at one hour post-injection, and then decreased to 0.115% after four hours, 0.02% after 24 hours, and 0.001% after 96 hours post-injection.

Although regulatory guideline acute and sub-chronic (13-week) neurotoxicity studies conducted on DQ at dose levels up to 150 mg DQ ion/kg bw/day provided no evidence of behavioral or neuropathological effects in male or female rats (Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment et al., 2013), those studies were limited because they did not specifically focus on neuropathology in the SNpc. Furthermore, the rat may not be a sensitive model for assessing the potential effects of chemical on the nigrostriatal dopaminergic system (Giovanni et al., 1994). Therefore, in the current study, the potential effects of DQ on the nigrostriatal dopaminergic system were assessed in male and female C57BL/6J mice exposed continuously to diquat dibromide ($DQ{\cdot}Br_2$) in their diets for 13 weeks using multiple (neuropathology, stereology, and neurochemistry) assessment endpoints.

2. Methods

2.1. Acute toxicokinetic study with DQ

An acute toxicokinetic study was conducted in the AAALAC-accredited (Association for Assessment and Accreditation of Laboratory Animal Care International) laboratory of Charles River Laboratories (Edinburgh, UK) in compliance with the UK Principles of Good Laboratory Practice (The Secretary of State (2004)). Thirty-two male C57BL/6J mice, aged eight to nine weeks, received a single ip injection of [^{14}C]-$DQ{\cdot}Br_2$ at a dose level of 1.42 mg $DQ{\cdot}Br_2$/kg bw (radioactive dose of 8.48 MBq/kg bw). Blood and brain samples were collected at 0.25, 0.5, 1, 2, 4, 24 and 48 hours post-dose (four mice/time point). A 200–300 μL aliquot of blood was collected into an EDTA blood tube immediately prior to euthanasia, and plasma was collected following centrifugation. The mice were then euthanatized by cervical dislocation and the brains were collected. Levels of radioactivity in the plasma and brain tissue were then determined by liquid scintillation counting (LSC).

2.2. 13-Week dietary study with DQ

The 13-week dietary study was conducted in the AAALAC-accredited laboratory of WIL Research (Ashland, OH) in compliance with Good Laboratory Practice Regulations (Organisation for Economic Co-operation and Development, 1998; United States Environmental Protection Agency, 1989). Brain neurochemistry investigations were performed by RTI International (Research Triangle Park, NC). Microscopic slides of brain sections for pathology evaluation were prepared by Neuroscience Associates, Inc. (Knoxville, TN) and examined at Tox Path Specialists, LLC (Frederick, MD) in randomized order by a pathologist who was "blinded" to treatment group. Brain tissues for stereological investigations were prepared at Experimental Pathology Laboratories (EPL, Sterling, VA) and evaluated in randomized order by an EPL stereologist who was "blinded" to treatment group.

2.2.1. Study design

The control and test diets were offered continuously for 13 weeks (91–95 consecutive days). Mice were assigned to four treatment groups (control, 12.5 ppm $DQ{\cdot}Br_2$, 62.5 ppm $DQ{\cdot}Br_2$ and 4 × 10 mg/kg bw/dose ip MPTP). Control and MPTP groups were provided a basal diet for the duration of the study. $DQ{\cdot}Br_2$ was administered in the diet as a constant concentration of either 12.5 or 62.5 ppm; these concentrations were selected to achieve dose levels of approximately 1.5 and 7.5 mg DQ (ion)/kg bw/day, respectively, over the 13-week period. These two $DQ{\cdot}Br_2$ exposure levels were adequately spaced to allow the observation of potential dose responses, and the top exposure level was expected to not exceed the maximum tolerated dose for mice of this strain (as based on the toxicity noted at 300 ppm [equating to approximately 43 mg/kg/day] in a chronic study using CD-1 mice; United States Environmental Protection Agency, 1995).

For mice designated for neurochemical and stereological assessments, MPTP was administered seven days prior to scheduled euthanasia, whereas mice designated for pathologic assessment were administered MPTP two days prior to scheduled euthanasia. MPTP was administered as four successive ip injections during Week 12; each at a dose of 10 mg (free base)/kg bw, spaced at approximately two-hour intervals, to provide a total dose of 40 mg/kg bw. This dose route and dosing regimen have been shown to produce a readily-discernable and reproducible lesion of the nigrostriatal dopaminergic pathway (Breckenridge et al., 2013; Jackson-Lewis and Przedborski, 2007). The dose volume for the MPTP group was 2.5 mL/kg bw/dose.

2.2.2. Preparation of diquat diets

Diets were prepared with $DQ{\cdot}Br_2$ monohydrate (Syngenta batch number 695099; lot 3D29-01) the purity of which was 96.5% (containing 49.1% as diquat cation). Prior to use, the $DQ{\cdot}Br_2$ was dried in an oven set at approximately 100 °C for a minimum of four hours. At each dietary concentration, the appropriate amount of $DQ{\cdot}Br_2$ was blended into a portion of the PMI Nutrition International, LLC Certified Rodent LabDiet® 5002 (meal). The resulting premix was then mixed thoroughly with the remaining amount of feed to obtain the appropriate dietary concentrations. Test diets were prepared at approximately weekly intervals. Analysis of dietary samples collected at various intervals throughout the study indicated that dietary $DQ{\cdot}Br_2$ concentrations ranged from 92.7 to 104% of target concentrations.

2.2.3. Preparation of MPTP dosing solution

MPTP·HCl was obtained from Sigma–Aldrich, Inc., St. Louis, MO (Batch Number SLBC4446V) and stored at room temperature. MPTP·HCl was formulated as solutions in physiological (0.9%) saline at a target concentrations of 4.0 (as free base)/mL. The concentrations of MPTP, as verified by HPLC, ranged from 104 to 108% of the target concentration.

2.2.4. Test animal/Animal husbandry

Male and female C57BL/6J mice, six weeks of age, were supplied by Jackson Laboratories, Bar Harbor, ME. Mice were housed in individual, stainless-steel, wire-mesh-floored cages. Individual body weight and food consumption data were recorded weekly for all animals. A 12-hour light–dark photoperiod was maintained throughout the study; room temperature and relative humidity were maintained at 22 °C ± 3 °C and 50% ± 20%, respectively. One

week prior to initiation of DQ treatment, the mice were assigned randomly to control and treatment groups using a randomized block design, stratified by weight. Treatment with diquat (or basal diet) commenced at approximately nine weeks of age and MPTP administration occurred when the mice were approximately 21 weeks of age.

2.2.5. Striatal neurochemistry

After 13 weeks of DQ·Br_2 exposure, or one week following MPTP administration, surviving mice designated for striatal neurochemistry were euthanized by cervical dislocation, followed by decapitation and brain removal. The striatal tissue was isolated, weighed and flash-frozen in liquid nitrogen. Striatal concentrations of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were determined by HPLC coupled with electrochemical detection, as described previously (Breckenridge et al., 2013). Dopamine turnover was calculated ([DOPAC + HVA] ÷ [DA]).

2.2.6. Striatal and SNpc neuropathology

Control, 12.5 ppm DQ·Cl_2, and 62.5 ppm DQ·Cl_2 mice designated for neuropathology assessment were euthanized after four, eight or 13 weeks of exposure. Surviving MPTP mice designated for neuropathology assessment were euthanized during Week 14 (two days following MPTP treatment). Mice were anesthetized (sodium pentobarbital ip) and perfused *in situ* with 25 mL of sodium cacodylate buffer, followed by perfusion with 75 mL of sodium cacodylate-based 4% paraformaldehyde (methanol-free). The heads were placed in cacodylate-based 4% paraformaldehyde for 24 h. The brains were then removed from the skulls, weighed, measured (length and width), placed into the sodium cacodylate buffer solution and maintained refrigerated. The brains were shipped to Neuroscience Associates, Inc., where they were trimmed, multi-embedded, and sectioned at 30 μm in the coronal plane. Serial sections were produced and stained using amino cupric silver (AmCuAg) stain (for neuronal necrosis), tyrosine hydroxylase (TH) immunostain (for dopaminergic neurons and processes), glial fibrillary acidic protein (GFAP) immunostain (for astrocytes), ionized calcium binding adaptor molecule 1 (IBA-1) immunostain (for microglia), caspase-3 immunostain (SNpc region only; for apoptosis), thionine stain (for general morphology) or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) stain (for apoptosis), as previously described (Breckenridge et al., 2013). All slides were shipped to Tox Path Specialists, LLC for examination. Microscopic examination was performed in a "blinded" manner; the pathologist was not aware of the treatment group to which the individual mouse brain sections belonged. Neuropathology data were recorded according to a semi-quantitative scoring system, as previously described (Breckenridge et al., 2013), including analysis of the presence of necrotic cells, the character of any glial reaction, an assessment of the degree of dopaminergic neuron loss (if any), and an assessment of the possible mechanism for the loss.

2.2.7. SNpc stereology

After 13 weeks of DQ·Br_2 exposure, or one week following MPTP, surviving mice designated for stereological assessment of the number of TH^+ neurons in the SNpc were anesthetized (sodium pentobarbital ip) and perfused *in situ* with chilled 0.9% physiological saline, followed by 4% paraformaldehyde (pH approximately 9.5), using a flow rate of approximately 10 mL/min. The perfusion-fixed brains (including olfactory bulbs) were preserved in 4% paraformaldehyde and then shipped to Experimental Pathology Laboratories, Inc. for further processing, sectioning and staining, as previously described (Breckenridge et al., 2013). The stereological assessment was performed in a "blinded" manner; the investigator

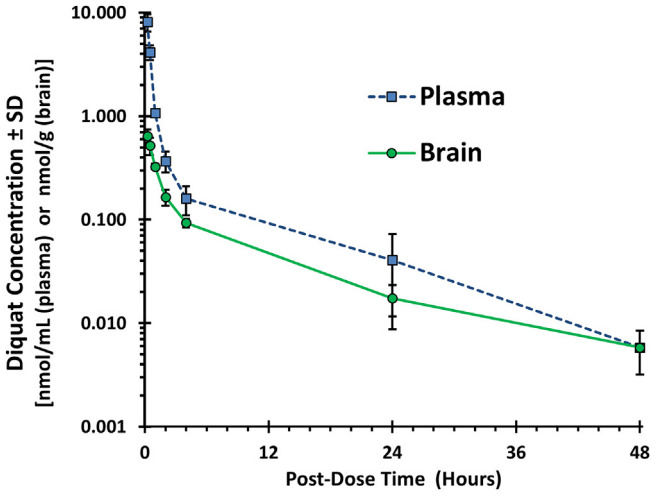

Fig. 1. Plasma and brain DQ concentrations following acute exposure. Mean plasma and brain DQ concentrations in mice are shown at various intervals following a single ip injection of [^{14}C]-diquat dibromide at a dose level of 1.42 mg DQ·Br_2/kg bw (n = 4 mice/time point).

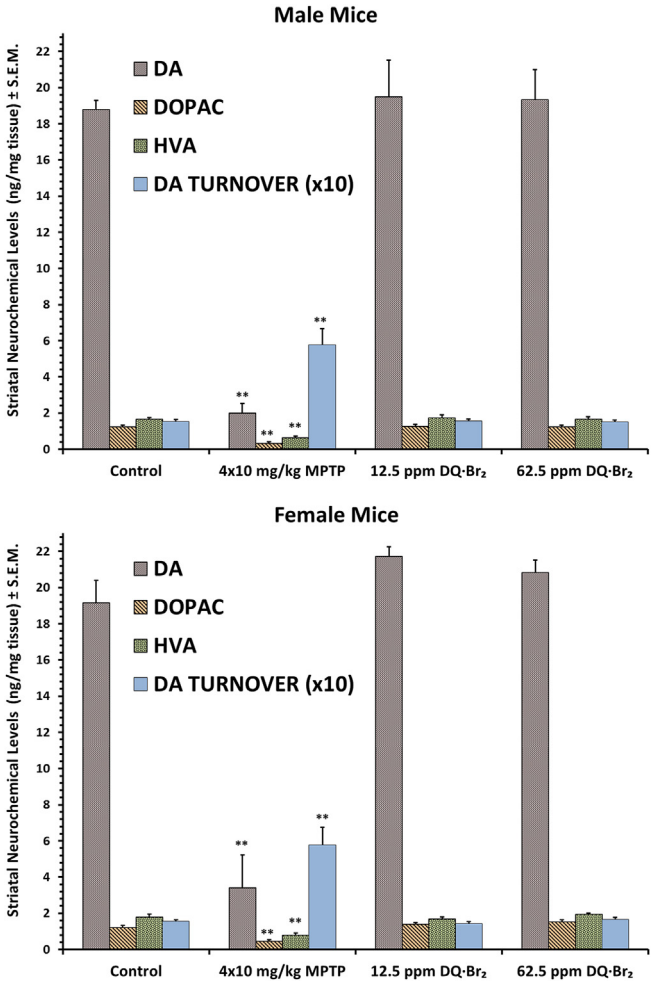

Fig. 2. Neurochemistry. Mean striatal dopamine (DA), DOPAC and HVA concentrations (ng/mg tissue) after 13 weeks of dietary treatment with DQ·Br_2 or seven days after the ip administration of MPTP. DA turnover (expressed as a ratio) is estimated according to the following formula: DA turnover = ([HVA] + [DOPAC])/[DA]. Sample size (n) = 6 mice/sex/group except for 12.5 ppm DQ·Br_2 females (n = 5). *p ≤ 0.05 **p ≤ 0.01.

was not aware of the treatment group assignment of individual mice.

The total number of TH^+ neurons in the SNpc was estimated using the optical fractionator approach (West et al., 1991), which employed an unbiased, systematic random-sampling methodology. Accordingly, neuron cell bodies were counted in a subsample of sections throughout the entire depth of the stained tissue, and then the results were extrapolated to provide estimates of total numbers of TH^+ neurons in the left and right SNpc. The following stereological parameters were used in this study: section sampling frequency = one of every three SNpc-containing sections (microtomed at 40 μm; approximately 10–12 sections total); counting frame interval = 200 × 200 μm; counting frame size = 60 × 60 μm; and disector height = entire depth of section.

2.2.8. Statistical analyses

Initial half-lives (t½) were determined based on an exponential fit of the mean plasma and brain concentrations from the first four time points (0.25, 0.5, 1 and 2 h post-dose), whereas terminal half-lives (t½) were determined based on an exponential fit of the mean plasma and brain concentrations from the last three time points (4, 24 and 48 h post-dose) using the formula $t½ = \ln(2)/-(\text{slope})$.

The effects of treatment on the concentrations of dopamine, dopamine metabolites (DOPAC, HVA) and dopamine turnover were analyzed using ANOVA. When the ANOVA analysis revealed significant differences ($p < 0.05$) in intergroup variance, Dunnett's test (Dunnett, 1964) was used to compare the mean values from DQ and MPTP groups to the control group mean values.

One-sided two-sample Welch's *t*-tests (Welch, 1947) were used to evaluate the null hypothesis of no treatment-related change in the mean number of TH^+ neurons in the SNpc of the MPTP and DQ-treated groups *versus* the alternative hypothesis of a treatment-related decrease in the mean number of TH^+ neurons in the SNpc, as compared to the control group. Differences in means were considered statistically significant at $p \leq 0.05$.

Group mean pathologic severity scores based on the categorical classification of microscopic findings were calculated by Tox Path Specialists, LLC. Statistically-significant mean severity score differences between the groups were determined using a standard Z score as the test statistic and compared to the standard normal distribution (*i.e.*, the normal distribution with mean 0 and variance 1) in a one-sided test at the 5% significance level.

3. Results

3.1. Brain and plasma levels following acute exposure

Following the single ip injection of [^{14}C]-DQ·Br_2 at a dose level of 1.42 mg DQ·Br_2/kg bw, both the plasma and brain DQ concentrations peaked at the earliest time point assessed (15 min) and declined rapidly thereafter (Fig. 1). The initial half-lives (t½) for plasma and brain were approximately 0.40 and 0.89 h, respectively. The terminal half-lives (t½) for plasma and brain were approximately 9.1 and 11.2 hours, respectively.

3.2. Achieved diquat dose levels following dietary exposure

The mean dose levels achieved during the 13-week study for the 12.5 and 62.5 ppm DQ·Br_2 groups were 2.6 and 13.0 mg/kg bw/day for males, and 3.4 and 15.5 mg/kg bw/day for females, respectively. The corresponding doses expressed as DQ ion were 1.3 and 6.4 mg DQ (ion)/kg bw/day for males, and 1.7 and 7.6 mg DQ (ion)/kg bw/day for females, respectively (weekly mean achieved dose levels are presented in Supplemental Fig. 1).

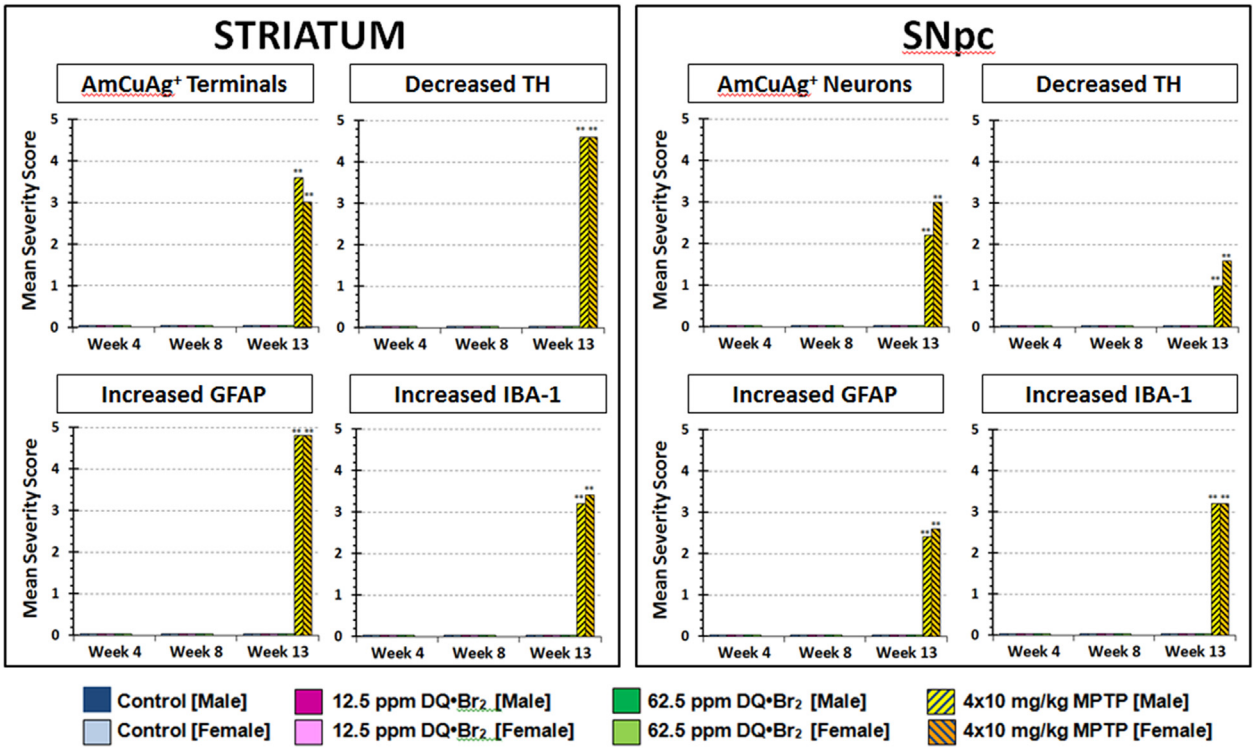

Fig. 3. Pathology severity scores. Mean pathology scores for the striatum and SNpc, based on the presence of silver positive staining (AmCuAg), decreased tyrosine hydroxylase staining, increased GFAP staining and increased IBA-1 staining after four, eight and 13 weeks of dietary treatment with DQ·Br_2, or two days after the ip administration of 40 mg/kg bw (4 × 10 mg/kg bw/dose) MPTP. Mean severity scores (based on numerical grading scores ranging from 0 [normal] to 5 [marked]) are based on four or five animals/sex/group at each assessment interval. $^{**}p \leq 0.01$.

3.3. Clinical observations, body weights and food consumption

There were no DQ·Br_2-related clinical findings noted during the study. Although two females in the 12.5 ppm DQ·Br_2 group were found dead on study days 67 and 71 respectively, these unscheduled deaths were not considered to be test-substance-related, since no mortality was noted at 62.5 ppm. Mean body weight gains were reduced compared to controls during the first week for the DQ·Br_2-treated males and females at 12.5 and 62.5 ppm, and during the second week in females at 12.5 and 62.5 ppm. These effects on mean body weight gain occurred in the absence of corresponding effects on food consumption. After the second week, mean body weights and body weight gains for males and females in DQ·Br_2 groups were generally comparable to the controls (weekly mean body weight and food consumption data are presented in Supplemental Fig. 1).

Within the first two days following MPTP treatment, six mice (four males and two females) were found dead. Clinical findings noted for MPTP-treated mice (including the surviving mice) on the day of dosing included hypoactivity, tremors, hunched posture, a cool body, half-closed eyelids, decreased respiration, increased respiration, and Straub (rigid erect) tail (incidence of clinical signs following MPTP dosing are presented in Supplemental Table 1). These clinical signs are typical effects of MPTP as injected ip in mice, and have been observed in previous studies employing this MPTP dosing regimen (Breckenridge et al., 2013; Minnema et al., 2014). None of these findings persisted to the time of scheduled termination. MPTP administration also resulted in a loss in mean body weight for males and females during the week following MPTP administration.

3.4. Striatal neurochemistry

There were no DQ-related effects on striatal dopamine concentrations, dopamine metabolite (DOPAC and HVA) concentrations or dopamine turnover (Fig. 2).

Treatment with MPTP at 40 mg/kg bw/dose (4 × 10 mg/kg bw/dose) resulted in statistically-significant decreases in striatal dopamine and dopamine metabolite (DOPAC and HVA) concentrations, and statistically-significant increases in dopamine turnover.

3.5. Striatal and SNpc Neurohistopathology

Dietary exposure to DQ for four, eight or 13 weeks did not result in any gross or microscopic changes in any of the brain regions,

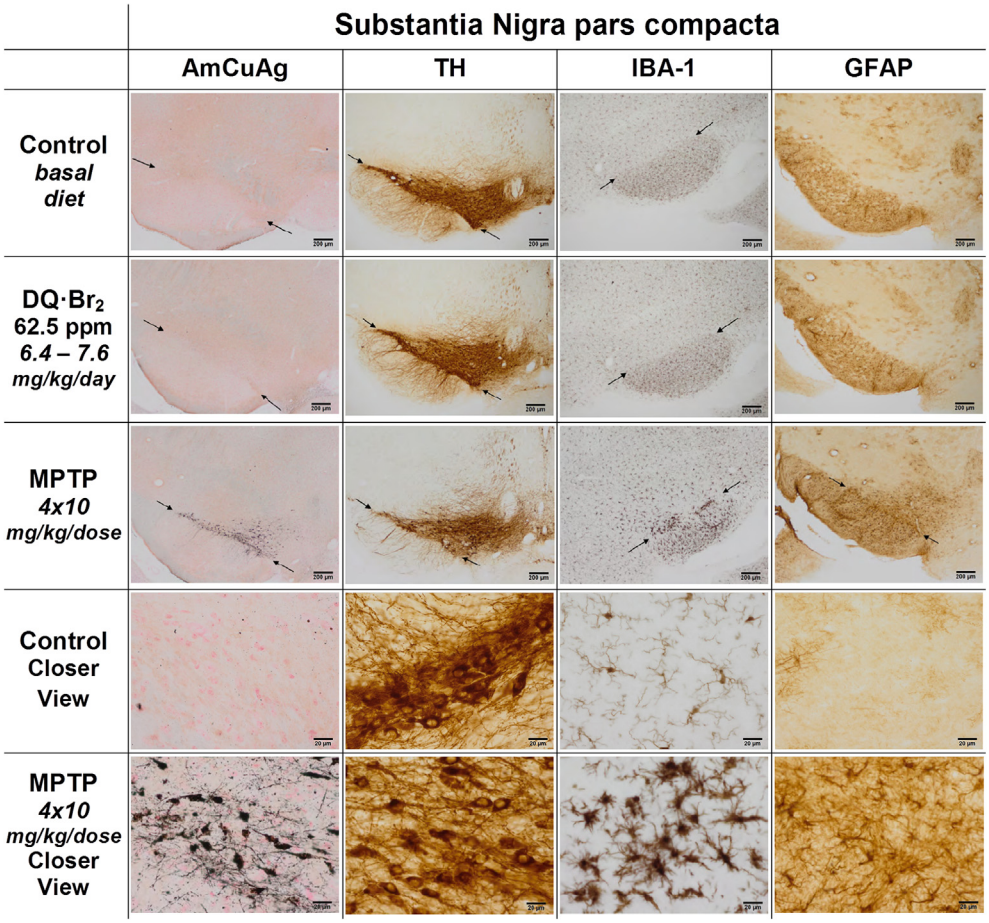

Fig. 4. Photomicrographs of the SNpc region from control, DQ·Br_2-exposed and MPTP-treated mice. Arrows in the photomicrographs indicate the region of the SNpc. No differences were noted in the SNpc of DQ·Br_2-exposed mice as compared to control mice. Two days after treatment, there were visible increases in silver (AmAgCu) staining in the SNpc region of mice treated with MPTP. Decreases in TH staining of neurons in the SNpc were observed for mice treated with MPTP. Note the lack of a linear area of dense TH^+ staining representing the dopaminergic neurons of the SNpc. There is pronounced and diffuse astrocytosis (as indicated by increased GFAP staining) in the SNpc and the *substantia nigra* pars reticulata (SNpr). Note that astrocytes are normally somewhat numerous in the SNpr. In the SNpc, there is a detectable linear increase of astrocyte staining in the SNpc from MPTP-treated mice, indicative of an increase of astrocytes/reactive astrocytes in the region of the SNpc, which is likely a response to neuronal loss. A linear increase of microglial cells in the SNpc, as indicated by the increased IBA-1 staining in this region (see arrows), was noted for the MPTP-treated mice.

from the level of the frontal cortex through the striatum and midbrain, including the SNpc (mean brain lengths and widths after 13 weeks of DQ exposure are presented in Supplemental Fig. 2). Brains of DQ-treated mice were indistinguishable from the brains of control mice. No morphologic changes attributable to DQ exposure were observed with the stains/immunostains that were used to evaluate neuronal cell death (Am Cu Ag); dopaminergic neurons/processes (DAB/TH); astrocytic response (GFAP); microglial response (IBA-1); apoptotic cell death (caspase-3); nuclear changes (thionine); or DNA fragmentation (TUNEL). Based on mean severity scores, there were no statistically-significant differences between the DQ-exposed groups vs. control groups with respect to neuronal, astrocytic or microglial changes in the SNpc or the striatum (Fig. 3). Representative sections of SNpc and striatum processed with AmCuAg stain, TH immunostain, GFAP immunostain and IBA-1 immunostain are shown in Figs. 4 and 5.

Pathologic findings attributed to MPTP included statistically-significant decreases in TH immunostaining, increases in silver (AmAgCu) staining, increases in GFAP immunostaining, and increases in IBA-1 immunostaining in both the SNpc and the striatum, relative to controls (Figs. 3–5). Two days after treatment, increased cupric silver staining, indicative of neurodegenerative changes, was noted in the striatum and SNpc of all MPTP-treated animals. Similarly, decreased TH immunostaining, consistent with damage to dopaminergic neurons, was noted in the striatum of all MPTP-treated animals, and in the SNpc for both 3/5 MPTP-treated males and 4/5 MPTP-treated females. Increased GFAP immunostaining (indicative of an increase in reactive astrocytes responding to neuronal injury) and increased IBA-1 immunostaining (indicative of a reactive microglial response to tissue damage) were noted in the striatum and SNpc for all MPTP-treated males and females. There was no evidence of MPTP-associated effects in brain tissues immunostained with caspase-3, thionine, or TUNEL (individual severity scores are presented in Supplemental Table 2).

3.6. SNpc TH^+ neuronal cell number

There were no statistically-significant effects of DQ in either male or female mice on the number of TH^+ neurons in the SNpc after 13 weeks of dietary exposure, at either 12.5 or 62.5 ppm DQ·Br_2 (Fig. 6).

One week following MPTP treatment, mean numbers of TH^+ neurons in the SNpc were decreased significantly, relative to controls, by 21.3% and 18.6% in male and female mice, respectively (Fig. 6).

4. Discussion

The data from the acute toxicokinetic study showed that following ip injection, DQ was very rapidly cleared from both the plasma and the brain, which is consistent with the more limited results of Lindquist (Lindquist et al., 1988). The percentage of DQ

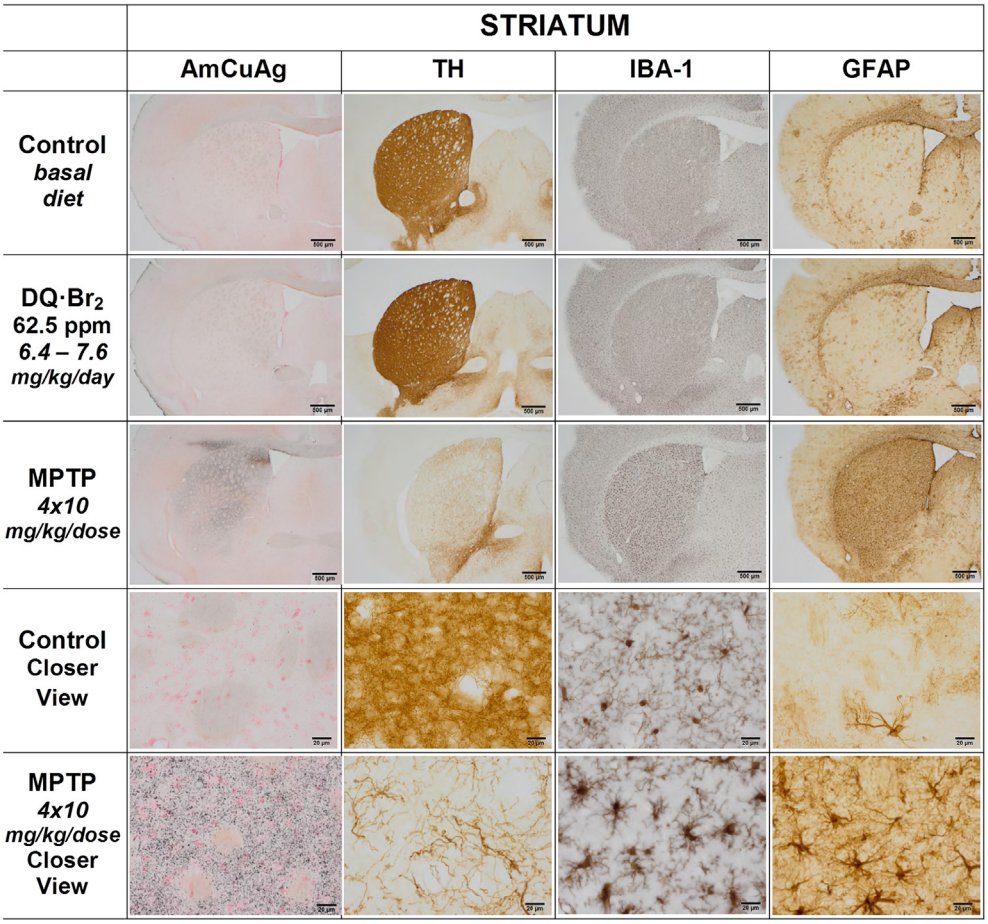

Fig. 5. Photomicrographs of the striatum from control, DQ·Br_2-exposed and MPTP-treated mice. No differences were observed in the striatum of DQ·Br_2 exposure mice as compared to control mice. Two days after 40 mg/kg bw (4 × 10 mg/kg bw/dose) MPTP treatment, there were notable increases in silver (AmAgCu) staining, GFAP immunostaining and IBA-1 immunostaining, as well as decreases in TH immunostaining in the striatum of MPTP-treated mice.

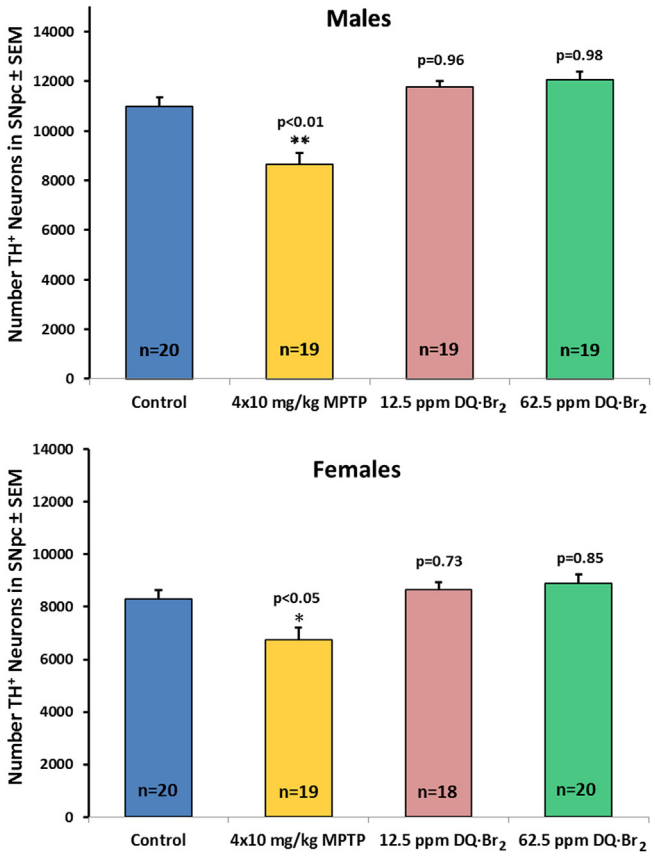

Fig. 6. SNpc stereology. Stereological assessment of the number of tyrosine hydroxylase positive (TH^+) neurons in the *substantia nigra* pars compacta (SNpc) after 13 weeks of dietary exposure to DQ·Br_2 or seven days after ip administration of 40 mg/kg bw (4 × 10 mg/kg bw/dose) MPTP. The numbers of mice assessed for each treatment group per sex are indicated on the graph. $^*p \leq 0.05$ $^{**}p \leq 0.01$.

present in the brain was, at most, 0.26% of the administered dose (15 min post-dose), and fell to 0.006% after 24 h. The rate of clearance of DQ from the brain, which is much more rapid than the terminal half-life of 24 days reported for PQ following an ip injection (Breckenridge et al., 2013), indicates that DQ has very limited potential for accumulation in the brain following continuous dietary exposure.

The results of the dietary study demonstrated that DQ was well-tolerated by the male and female C57BL/6J mice that were exposed continuously to DQ·Br_2 for 13 weeks, as indicated by only an initial transient reduction in body weight gain at both dietary concentrations. Prolonged exposure to DQ·Br_2 at dietary concentrations up to 62.5 ppm (achieved dose levels of 6.4 and 7.6 mg DQ [ion]/kg bw/day for males and females, respectively) did not result in any effects on the nigrostriatal dopaminergic system. Specifically, exposure to DQ did not result in a reduction in the number of TH^+ dopaminergic neurons in the SNpc, did not alter the concentration of striatal dopamine or its metabolites, and did not produce evidence of neuronal cell damage or glial activation. The achieved dose levels are approximately 13–15 times the current chronic reference (point of departure) dose of 0.5 mg DQ (ion)/kg bw/day (Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment et al., 2013; United States Environmental Protection Agency, 2009).

The DQ-exposed mice in the current 13-week study achieved total weekly oral dose levels of approximately 45–53 mg DQ (ion)/kg bw/week (male and females, respectively). Due to the different routes of administration, it is difficult to compare the systemic exposure to DQ in the current study to that of Karuppagounder (Karuppagounder et al., 2012), in which DQ was administered to C57BL/6 mice via ip injection at 20 mg/kg bw/week (10 mg/kg bw/dose, twice weekly, for six weeks). The significant reduction of body weight gain reported by Karuppagounder and colleagues suggests that the systemic DQ dose achieved in their study was greater than that achieved in the current study. Those investigators reported that, at the time of behavioral testing, the mean body weight of the DQ-treated mice was 13.3% less than that of the control group; the extent of this reduced body weight gain is suggestive of an overall deterioration in health status. The impact of this relatively marked decrease in body weight gain on the various assessment parameters is unknown. Those investigators reported no effect of DQ treatment on striatal dopamine concentration; however, a decrease in the mean striatal DOPAC concentration was noted. Considered in isolation, the toxicological significance of that finding (if any) is unclear. Karuppagounder et al. also reported that immunohistochemical staining for TH^+ neurons in the SN was reduced, which they considered to be indicative of a qualitative reduction in the number of TH^+ neurons. However, the authors did not state whether the pars compacta region alone was examined or whether the whole substantia nigral region was assessed. Furthermore, in contrast to our own study, the evidence for this reported reduction in TH^+ immunostaining in the SN was presented in the form of representative control and DQ-treated micrographs; the extent of any neuronal cell loss was not quantified, either by stereology or TH staining densitometry. Additionally, there was no indication in the Karuppagounder et al. paper that the qualitative assessment of TH^+ immunostaining was conducted in a randomized, blinded manner in order to eliminate the possibility of observational bias.

Overall, the null results obtained in the blinded neurochemical, histopathological and stereological evaluations of our DQ study conducted in a sensitive strain of mice (Hamre et al., 1999; Yin et al., 2011) are comparable to those obtained for PQ following dietary (Minnema et al., 2014) or ip (Breckenridge et al., 2013) administration. If neurons in the SNpc died or were undergoing neuropathological changes as a result of toxicity caused by continuous exposure to DQ for 13 weeks, one would have expected that sensitive indicators of neurotoxicity in the SNpc and/or striatum would have been apparent. In contrast, we found: 1) no evidence of reduced numbers of TH^+ dopaminergic neurons in the SNpc based upon unbiased stereological methods; 2) no evidence of decreased staining of TH^+ neurons in the SNpc or striatum assessed qualitatively by a histopathologist blinded to dose group; 3) no histopathological evidence of cell death (apoptosis, silver stain uptake) or reactive astrocytosis or gliosis; and 4) no changes in dopamine concentration in the striatum. In contrast to DQ, we observed this constellation of effects, which are indicative of damage to the nigrostriatal dopaminergic system, in MPTP-treated male and female mice.

Conflict of interest statement

These studies were funded by Syngenta Crop Protection, LLC, a registrant and basic manufacturer of diquat dibromide. All authors of this review either worked for the institutes where the research was conducted (DZ, JCW, SLW, MTH and MB) or are employees of Syngenta, LLC (ARC, CBB, DJM, KZT, NCS and PAB).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.yrtph.2015.12.001.

Transparency document

Transparency document related to this article can be found online at doi:10.1016/j.yrtph.2015.12.001.

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